



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/GB91/01632 <b>(22) International Filing Date:</b> 23 September 1991 (23.09.91) <b>(30) Priority data:</b> 9020799.4                      25 September 1990 (25.09.90) GB 9104903.1                      8 March 1991 (08.03.91) GB <b>(71) Applicant (for all designated States except US):</b> IMMUNOL- OGY LIMITED [GB/GB]; 184 Cambridge Science Park, Milton Road, Cambridge CB4 4GN (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> INGLIS, Stephen, Charles [GB/GB]; BOURSNEILL, Michael, Edward, Griffith [GB/GB]; Immunology Limited, 184 Cambridge Science Park, Milton Road, Cambridge CB4 4GN (GB). MINSON, Anthony, Charles [GB/GB]; 113 Cambridge Road, Great Shelford, Cambridge CB2 5JJ (GB).		<b>(74) Agent:</b> MEWBURN ELLIS; 2 Cursitor Street, London EC4A 1BQ (GB). <b>(81) Designated States:</b> AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (Eu- ropean patent), GN (OAPI patent), GR (European pa- tent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European pa- tent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU <sup>+</sup> , TD (OAPI patent), TG (OAPI pa- tent), US. <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> VIRAL DEFECTIVE VACCINE PRODUCED BY TRANSCOMPLEMENTING CELL LINE  <b>(57) Abstract</b> <p>A mutant virus for use as a vaccine, wherein the genome of the virus is defective in respect of a gene essential for the pro-          duction of infectious virus. In one aspect the mutant virus is capable of protecting a susceptible species immunized therewith          against infection by the corresponding wild-type virus. In another aspect, the mutant virus acts as a vector for an immunogenic          protein derived from a pathogen and which is encoded by foreign DNA incorporated in the mutant virus. The mutant virus can          be produced in a recombinant host cell which expresses a gene complementing the defect. The mutant virus is preferably infec-          tious for the host to be protected, but the defective gene allows expression in the infected host of at least some of the viral genes,          which can provoke a cell-mediated immune response.</p>		

# + DESIGNATIONS OF "SU"

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### Viral defective vaccine produced by transcomplementing cell line

The present invention relates to viral vaccines. In particular, it relates to genetically engineered  
5 mutant viruses for use as vaccines; vaccines comprising the mutant viruses; recombinant cell; and to methods relating to the production of vaccines.

Viral vaccines are traditionally of two sorts. The  
10 first sort are 'killed' vaccines, which are virus preparations which have been killed by treatment with a suitable chemical such as beta-propiolactone. The second type are live 'attenuated' vaccines, which are viruses which have been rendered less pathogenic to  
15 the host, either by specific genetic manipulation of the virus genome, or, more usually, by passage in some type of tissue culture system. These two types of vaccine each have their own disadvantages. Since killed vaccines do not replicate in the host, they  
20 must be administered by injection, and hence may generate an inappropriate kind of immune response. For example the Salk vaccine, a killed preparation of poliovirus, produces an immunoglobulin (Ig) G antibody response, but does not stimulate the production of IgA  
25 in the gut, the natural site of primary infection. Hence this vaccine, though it can protect the

individual from the neurological complications of poliomyelitis, does not block primary infection, and so does not confer "herd immunity". In addition, killed viruses, do not enter and replicate inside host  
5 cells. Hence any beneficial immunological response to non-structural proteins produced during replication is not available. They also cannot stimulate the production of cytotoxic T cells directed against virus antigens. "Dead" antigens can be picked up by antigen  
10 presenting cells and presented to T cells. However, the presentation occurs via MHC Class II molecules and leads to stimulation of T helper cells. In turn, the T helper cells help B cells to produce specific antibody against the antigen. In order to stimulate  
15 the production of cytotoxic T cells, virus antigens must be processed through a particular pathway inside the infected cell, and presented as broken-up peptide fragments on MHC Class I molecules. This degradation pathway is thought to work most effectively for  
20 proteins that are synthesised inside the infected cell, and hence only virus that enters host cells and expresses immunogenic viral protein is capable of generating virus-specific cytotoxic T cells. Therefore, killed vaccines are poor inducers of  
25 cellular immunity against virus infection. From this point of view, live attenuated vaccines are more

satisfactory.

To date, live attenuated viruses have been made by deleting an inessential gene or partly damaging one or more essential genes (in which case, the damage is such that the genes are still functional, but do not operate so effectively). However, live attenuated viruses often retain residual pathogenicity which can have a deleterious effect on the host. In addition, unless the attenuation is caused by a specific deletion, there remains the possibility of reversion to a more virulent form. Nevertheless, the fact that some viral protein production occurs in the host means that they are often more effective than killed vaccines which cannot produce such viral protein.

Live attenuated viruses, as well as being used as vaccines in their own right, can also be used as 'vaccine vectors' for other genes, in other words carriers of genes from a second virus (or other pathogen) against which protection is required. Typically, members of the pox virus family eg. vaccinia virus, are used as vaccine vectors. When a virus, is used as a vaccine vector, it is important that it causes no pathogenic effects. In other words it may need to be attenuated in the same way that a

simple virus vaccine is attenuated. The same disadvantages as those described above, therefore apply in this case.

- 5 It has been found possible to delete a gene from a viral genome and provide a so-called 'complementing' cell which provides the virus with the product of the deleted gene. This has been achieved for certain viruses, for example adenoviruses, herpesviruses and  
10 retroviruses. For adenoviruses, a human cell line was transformed with fragments of adenovirus type 5 DNA (Graham, Smiley, Russell & Nairn, J. Gen. Virol., 36,59-72, 1977). The cell line expressed certain viral genes, and it was found that it could support  
15 the growth of virus mutants which had those genes deleted or inactivated (Harrison, Graham & Williams, Virology 77, 319-329, 1977). Although the virus grew well on this cell line (the 'complementing cell line') and produced standard viral particles, it could not  
20 grow at all on normal human cells. Cells expressing the T-antigen-encoding region of the SV40 virus genome (a papovavirus) have also been shown capable of supporting the replication of viruses specifically deleted in this region (Gluzman, Cell, 23,182-195,  
25 1981). For herpes simplex virus, cell lines expressing the gB glycoprotein (Cai et al, J. Virol. 62,714-721,

1987) the gD glycoprotein (Ligas and Johnson, J. Virol. 62,1486, 1988) and the Immediate Early protein ICP4 (Deluca et al., J. Virol., 56,558, 1985) have been produced, and these have been shown capable of supporting the replication of viruses with specifically inactivated copies of the corresponding genes.

The present invention provides a mutant virus for use as a vaccine, in which a viral gene encoding a protein which is essential for the production of infectious virus has been deleted or inactivated; and wherein said virus can be grown in a cell which has a heterologous nucleotide sequence which allows said cell to express the essential protein encoded by said deleted or inactivated viral gene.

The present invention also provides a vaccine which comprises a virus as described above, together with one or more excipients and/or adjuvants. The viral genome may itself provide the immunogen, or it may contain a heterologous gene insert expressing the immunogenic protein.

The present invention also provides a complementing cell transfected with an attenuated virus as described

above, for use in the preparation of a vaccine.

The present invention also provides a method which comprises the use of a virus as described above in the  
5 preparation of a vaccine for the therapeutic or prophylactic treatment of a disease.

The present invention also provides a method for the production of a vaccine which comprises: culturing a  
10 cell infected with a virus having a deleted or inactivated viral gene encoding a protein which is essential for the production of infectious virus, and wherein the host cell has a heterologous nucleotide sequence comprising said viral gene and which is able  
15 to express the essential protein encoded by said gene; harvesting the virus thus produced, and using it in a vaccine.

The virus may be derived from herpes simplex virus  
20 (HSV) in which, for example, the gene encoding glycoprotein H (gH) has been inactivated or deleted. The mutant virus may also comprise a heterologous sequence encoding an immunogen derived from a pathogen. The host cell will suitably be a  
25 recombinant eukaryotic cell line containing the gene encoding HSV glycoprotein H. As another example the



virus may be derived from an orthopox virus, for example, vaccinia virus, which again may comprise a heterologous sequence encoding an immunogen derived from a pathogen.

5

This invention shows a unique way of combining the efficacy and safety of a killed vaccine with the extra immunological response induced by the in vivo production of viral protein by the attenuated vaccine.

10 In preferred embodiments it comprises two features. Firstly, a selected gene is inactivated within the virus genome, usually by creating a specific deletion. This gene will be involved in the production of infectious virus, but preferably not preventing  
15 replication of the viral genome. Thus the infected cell can produce more viral protein from the replicated genetic material, and in some cases new virus particles may be produced, but these would not be infectious. This means that the viral infection  
20 cannot spread from the site of inoculation.

A second feature of the invention is a cell which provides the virus with the product of the deleted gene, thus making it possible to grow the virus in  
25 tissue culture. Hence, although the virus lacks a gene encoding an essential protein, if it is grown in the

appropriate host cell, it will multiply and produce complete virus particles which are to outward appearances indistinguishable from the original virus. This mutant virus preparation is inactive in the sense  
5 that it has a defective genome and cannot produce infectious virus in a normal host, and so may be administered safely in the quantity required to generate directly a humoral response in the host. Thus, the mutant virus need not be infectious for the  
10 cells of the host to be protected and merely operates in much the same way as a conventional killed or attenuated virus vaccine. However, preferably the immunising virus is itself still infectious, in the sense that it can bind to a cell, enter it, and  
15 initiate the viral replication cycle and is therefore capable of initiating an infection within a host cell of the species to be protected, and producing therein some virus antigen. There is thus the additional opportunity to stimulate the cellular arm of the host  
20 immune system.

The deleted or inactivated gene is preferably one involved as late as possible in the viral cycle, so as to provide as many viral proteins as possible in vivo  
25 for generating an immunogenic response. For example, the gene may be one involved in packaging or some

other post-replicative event, such as the gH glycoprotein of HSV. However, the selected gene may be one involved in the viral genome replication, and the range of proteins expressed in vivo will depend upon the stage at which that gene is normally expressed. In the case of human cytomegalovirus (HCMV) the selected gene may be one (other than the Immediate Early gene) that effectively prevents viral genome replication in vivo, since the Immediate Early gene which is produced prior to viral genome replication (and indeed is essential for it) is highly immunogenic.

This invention can be applied to any virus where one or more essential gene(s) can be identified and deleted from or inactivated within the virus genome. For DNA viruses, such as Adeno, Herpes, Papova, Papilloma and Parvo viruses, this can be achieved directly by (i) the in vitro manipulation of cloned DNA copies of the selected essential gene to create specific DNA changes; and (ii) re-introduction of the altered version into the virus genome through standard procedures of recombination and marker rescue. The invention however, is also applicable to RNA viruses. Techniques are now available which allow complementary DNA copies of a RNA virus genome to be manipulated in

vitro by standard genetic techniques, and then converted to RNA by in vitro transcription. The resulting RNAs may then be re-introduced into the virus genome. The technique has been used to create  
5 specific changes in the genome of both positive and negative stranded RNA viruses, e.g. poliovirus (Racaniello and Baltimore, Science, 214, 916-919, 1981) and influenza virus (Lutyes et al., Cell, 59, 1107-1113, 1989).

10

In theory, any gene encoding an essential protein should be a potential target for this approach to the creation of attenuated viruses. In practice however, the selection of the gene will be driven by a number  
15 of considerations.

1. The gene should preferably be one which is required later in infection. Thus replication of the attenuated virus is not interrupted in the  
20 early phase. This means that most and possibly all other virus antigens will be produced in the infected cell, and presented to the host immune system in conjunction with host cell MHC class 1 molecules. Such presentation leads to the  
25 development of cellular immunity against virus infection through the production of cytotoxic T

cells. The cytotoxic T cells can recognise these antigens, and therefore kill virus infected cells. It is possible that the deleted gene could represent one which is not required at all for virus assembly, but is necessary for the assembled virus to be able to infect new cells. An example of such a protein is the HSV gH protein. In the absence of this protein, HSV virions are still produced, but they are non-infectious.

2. Ideally, the product of the selected gene should not, on its own, be toxic to the eukaryotic cell, so that a complementing cell can be produced relatively easily. This however is not an absolute requirement, since the gene may be placed under the control of an inducible promoter in the complementing cell, such that its expression may be switched on only when required.

20

The nature of the mutation created in the target gene is also a matter of choice. Any change which produces a non-functional gene product is satisfactory, as long as the risk of reversion to a wild type structure is minimised. Such changes include interruption of the target with extraneous sequences and creation of

25

specific deletions. The most satisfactory strategy for a vaccine to be used as a therapeutic and/or prophylactic however, would be one where a deletion is made that encompasses the entire sequence to be introduced into the complementing cell. The approach minimises the risk of regenerating wild type virus through recombination between the virus and cell DNA in the complementing cell.

10 Although there are several examples of combinations of specifically inactivated viruses and complementing cells, (see earlier discussion), to date, these have been used either for basic research on the virus, or, as in the case of retroviruses, to make a safer vector for producing transgenic animals. They have not been used for vaccine purposes, and to the applicants knowledge no suggestion of this kind of use has been proposed.

20 As well as using such an inactivated virus/complementing cell combination to produce safe vaccines against the wild-type virus, this invention also deals with the use of the same system to produce safe viral vectors for use as vaccines against foreign pathogens.

25

An example of such a vector is one based on HSV. The HSV genome is large enough to accommodate considerable additional genetic information and several examples of recombinant HSV viruses carrying and expressing foreign genetic material have been described (e.g. Ligas and Johnson, J. Virol. 1988, op. cit.). Thus a virus with a deletion in an essential virus gene as described above, and also carrying and expressing a defined foreign gene, could be used as a safe vector for vaccination to generate an immune response against the foreign protein.

A particular characteristic of HSV is that it may become latent in neurones of infected individuals, and occasionally reactivate leading to a local lesion. Thus an HSV with a deletion in an essential virus gene and expressing a foreign gene could be used to produce deliberately latent infection of neurones in the treated individual. Reactivation of such a latent infection would not lead to the production of a lesion, since the virus vector would be unable to replicate fully, but would result in the onset of the initial part of the virus replication cycle. During this time expression of the foreign antigen could occur, leading to the generation of immune response. In a situation where the deleted HSV gene specified a

protein which was not needed for virus assembly, but only for infectivity of assembled virions, such a foreign antigen might be incorporated into the assembled virus particles, leading to enhancement of its immunogenic effect. This expression of the foreign gene and incorporation of its protein in a viral particle could of course also occur at the stage where the mutant virus is first produced in its complementing host, in which case the mutant virus when used as a vaccine could present immediately the foreign protein to the species being treated.

In another example, vaccinia virus, a poxvirus, can carry and express genes from various pathogens, and it has been demonstrated that these form effective vaccines when used in animal experimental systems. The potential for use in humans is vast, but because of the known side effects associated with the widespread use of vaccinia as a vaccine against smallpox, there is reluctance to use an unmodified vaccinia virus on a large scale in humans. There have been attempts to attenuate vaccinia virus by deleting non-essential genes such as the vaccinia growth factor gene (Buller, Chakrabarti, Cooper, Twardzik & Moss, J. Virology 62,866-874, 1988). However, such attenuated viruses can still replicate in vivo, albeit at a



reduced level. No vaccinia virus with a deletion in an essential gene has yet been produced, but such a virus, deleted in an essential gene as described above, with its complementing cell for growth, would  
5 provide a safer version of this vaccine vector.

A further advantage of this general strategy for immunisation against heterologous proteins is that it may be possible to perform multiple effective  
10 vaccinations with the same virus vector in a way not possible with conventional live virus vectors. Since a standard live virus vaccine probably relies for its efficacy on its ability to replicate in the host animal through many cycles of infection, its  
15 usefulness will be severely curtailed in an individual with immunity against that virus. Thus a second challenge with the same virus, whether to provide a booster immunisation against the same protein, or a new response against a different protein, is likely to  
20 be ineffective. Using a virus vector with a deletion in an essential gene however, where multi-cycle replication is not desired or required, the events leading to effective immunisation will occur very soon after immunisation. The dose of the mutant virus can  
25 be relatively large (since it should be completely safe), and it is therefore unlikely that these early

events will be blocked by the host immune response, which will require some time to be mobilised completely.

5 Although we have referred above to a mutant virus being defective in an essential gene, and optionally containing a gene for an immunogenic pathogen protein, the mutant could be defective in more than one essential gene, and/or contain more than one  
10 immunogenic pathogen protein gene. Thus, the mutant virus might include the gene for HIV gp 120, to act as a vaccine in the manner suggested above, and also the gene for the HIV gag protein to be expressed within the vaccinated host and presented at the surface of  
15 the host cell in conjunction with MHC-I to stimulate a T-cell response in the host.

In order that the invention is more clearly understood, it will be further described by way of  
20 example only, and not by way of limitation, with reference to the following figures in which:

Fig.1 illustrates the production of plasmid pGH1;

Fig.2 illustrates the production of plasmid pGH2;

25 Fig.3a shows the pair of complementary oligonucleotides used to generate the plasmid pSP64Ta;

Fig.3b illustrates the production of plasmid pSP64TA;  
Fig.4a shows the two oligonucleotides used to generate  
the plasmid pCMVIEP;  
Fig.4b illustrates the plasmid pCMVIEP;  
5 Fig.5 illustrates the plasmid pCMVlacZ; and  
Fig.6 illustrates the plasmid pGH3.  
Fig.7 illustrates the strategy for construction of  
plasmid pGH-120.

10 Herpes Simplex Virus Deleted in Glycoprotein H (gH-  
HSV)

Herpes simplex virus (HSV) is a large DNA virus which  
causes a wide range of pathogenic symptoms in man,  
15 including recurrent facial and genital lesions, and a  
rare though often fatal encephalitis. Infection with  
this virus can be controlled to some extent by  
chemotherapy using the drug Acyclovir, but as yet  
there is no vaccine available to prevent primary  
20 infection. A difficulty with vaccination against HSV  
is that the virus generally spreads within the body by  
direct transfer from cell to cell. Thus humoral  
immunity is unlikely to be effective, since  
circulating antibody can only neutralise extracellular  
25 virus. Of more importance for the control of virus  
infection, is cellular immunity, and so a vaccine

which is capable of generating both humoral and cellular immunity, but which is also safe, would be a considerable advantage.

5 A suitable target gene for inactivation within the HSV genome is the glycoprotein H gene (gH). The gH protein is a glycoprotein which is present on the surface of the virus envelope. This protein is thought to be involved in the process of membrane  
10 fusion during entry of the virus into the infected cell. This is because temperature sensitive virus mutants with a lesion in this gene are not excreted from virus infected cells at the non-permissive temperature (Desai et al., J. Gen. Virol. 69,  
15 1147-1156, 1988). The protein is expressed late in infection, and so in its absence, a considerable amount of virus protein synthesis may still occur.

All genetic manipulation procedures are carried out  
20 according to standard methods described in "Molecular Cloning", A Laboratory Manual, eds. Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory Press, 1989.

A. Generation of a Cell line expressing the HSV gH gene

The gH gene is present in the Unique Long region (U<sub>L</sub>)  
5 of the HSV type 1 genome, between nucleotides 46382  
and 43868 (McGeoch et al, J. Gen. Virol. 69,  
1531-1574, 1988). A cloned copy of this gene is  
available within the plasmid pAF2. This plasmid was  
produced by excising a BgIII-XhoI fragment,  
10 encompassing the complete gH coding sequence, from the  
plasmid pTZgH, and cloning it into the BgIII site of  
plasmid pSP64T as described (Gompels and Minson, J.  
Virol., 63, 4744-4755, 1989). A HindIII fragment  
containing the promoter sequence for the glycoprotein  
15 D (gD) gene (extending from nucleotides -392 to +11,  
with respect to the start of the gD gene) is then  
excised from the plasmid pSVD4 (Everett, Nucl. Acids  
Res., 11, 6647-6667, 1983), and cloned into the unique  
HindIII site of pAF2 to generate pGH1 (figure 1) such  
20 that the promoter sequence is in the correct  
orientation to drive expression of the gH gene. Thus  
this plasmid contains the complete gH coding sequence  
under the control of the HSV type 1 gD gene promoter.  
This plasmid is then purified and then co-transfected  
25 into Vero cells with the plasmid pNEO (Pharmacia LKB  
Biotechnology Inc.) using the standard calcium

20

phosphate co-precipitation technique (Graham and Van  
der Eb, Virology 52, 456-467, 1973). Vero cells which  
have acquired resistance to neomycin are then selected  
by passage of the cells in the drug G418, and colonies  
5 of these cells cloned by limiting dilution. These  
neomycin resistant cells are then amplified in tissue  
culture, and samples are then infected with HSV type 2  
virus. Infection with the HSV type 2 virus has the  
effect of inducing transcription from the type 1 gD  
10 promoter present in the complementing cell genome, and  
so of stimulating production of the type 1 gH protein  
in the complementing cell. Lysates of the infected  
cells are then screened for expression of the gH  
protein by western blotting, using a polyclonal  
15 antiserum known to recognise specifically the type 1  
gH protein (Desai et al., 1988 op cit.). Cells which  
express the required protein are then retained and  
frozen stocks prepared. This material represents the  
gH+ complementing cell line.

20

B. Production of HSV type 1 virus with an interrupted  
gH gene

25 A 6432 base pair BgIII fragment containing the coding  
sequence of gH together with HSV flanking sequences is

excised from the plasmid pUG102 (Gompels and Minson, Virology 153, 230-247, 1986) and cloned into the plasmid pAT153 (Twigg and Sherratt, Nature, 283,216, 1980) to generate pGH2 (figure 2). This plasmid is  
5 digested with PvuII which cuts only within the gH coding sequence at two positions (nucleotides 44955 and 46065 according to the numbering scheme of McGeoch et al, 1988, op cit.), and the larger of the two fragments purified. A fragment of DNA consisting of  
10 the complete B-galactosidase gene from E coli downstream of the Immediate Early gene promoter from Cytomegalovirus (CMV) is then prepared by the following procedure. First of all a pair of complementary oligonucleotides (shown in figure 3a)  
15 are annealed and ligated with BgIII-digested, phosphatase-treated pSP64T (Krieg and Melton, Nucl. Acids Res. 12, 7057-7071, 1984) to generate the plasmid pSP64Ta as shown in figure 3b. The added linker also includes the initiation codon and first  
20 three codons of the B-galactosidase gene (lacZ) of E.coli. Next the "core region" of the Immediate Early gene promoter of CMV is amplified from plasmid pUG-H1 (Gompels and Minson, 1989, op cit.) by the Polymerase Chain Reaction technique (PCR-Molecular Cloning, ed.  
25 Sambrook et al., op cit.) using the two oligonucleotides shown in figure 4a, which correspond

to sequences from -302 to -288, and from -13 to -36 respectively (numbered in relation to the start of the CMV Immediate Early gene as described by Akrigg et al., Virus Research, 2, 107-121, 1985). These  
5 oligonucleotides also contain, at their 5'ends, sites for the restriction enzyme HindIII, and in the case of the oligonucleotide annealing upstream of the promoter, an additional SmaI site. The PCR-amplified product DNA is then digested with HindIII, and cloned  
10 into HindIII-digested pSP64Ta, to generate the plasmid pCMVIEP (figure 4b). Finally, a DNA fragment containing a complete copy of the E.coli B-galactosidase gene, lacking only the extreme 5'end of the coding sequence, is isolated by digestion of  
15 the plasmid pSC8 (Chakrabarti et al., Mol. Cell. Biol., 5, 3403-3409, 1985) with BamHI, and cloned into the unique BgIII site of pCMVIEP to generate pCMVlacZ (figure 5). A fragment of DNA containing the B-galactosidase gene under the control of the CMV IE  
20 promoter is then isolated by digestion of pCMVlacZ with SmaI, and ligated with the purified PvuII fragment of pGH2 described above, to generate pGH3, which consists of a copy of the gH gene interrupted by a functional B-galactosidase gene (figure 6). The  
25 next step is to replace the wild type gH gene in the HSV genome with this interrupted version, and this is



done by allowing recombination between HSV DNA and plasmid pGH3, followed by selection of those viruses which have acquired a functional B-galactosidase gene. Plasmid pGH3 DNA is therefore cotransfected into cells  
5 expressing the gH gene (the gH+ complementing cell line described in section A) along with purified HSV DNA isolated from purified HSV virions (Killington and Powell, In "Techniques in Virology: A practical Approach" (ed. B.W.J. Mahy) pp. 207-236, IRL Press,  
10 Oxford (1985)) by the standard calcium phosphate precipitation technique (Graham and Van der Eb, 1973, op cit.) The progeny HSV virus produced from this transfection experiment is then plated on monolayers of gH+ complementing cells by standard plaque assay,  
15 using an agar overlay, in the presence of 5-bromo-chloro-3-indolyl- $\beta$ -D-galactoside (X-gal), a chromogenic substrate which is converted to a blue substance by the enzyme  $\beta$ -galactosidase. Thus plaques resulting from infection by virus genomes containing  
20 and expressing the B-galactosidase gene will appear blue. These virus genomes should therefore carry an interrupted version of the gH gene. Virus is recovered from these plaques by picking plugs of agar from the appropriate part of the plate, and virus  
25 stocks prepared through growth of virus in the gH+ complementing cell line. These viruses, since they

bear non-functional versions of the gH gene, should be unable to form plaques on cells which do not contain and express an endogenous functional copy of the gH gene, and so to confirm this, a sample of the virus is  
5 assayed for its ability to form plaques on wild type Vero cell monolayers in comparison with the gH-complementing cells. Finally, virus DNA is prepared from these stocks, and checked for the expected DNA structure around the gH gene by Southern  
10 blotting. After confirmation of the correct genetic structure, a large stock of the gH gene-deficient virus is then prepared by inoculation of a sample of the virus into a large-scale culture of the gH+ complementing cell line (multiplicity of infection =  
15 0.01), and three days later, the infected cells are harvested. The infected cells are disrupted by sonication in order to release the cell-associated virus, and the total sonicated mixture stored at -70° as the virus master stock. The titre of the virus  
20 stock is then established by plaque assay on the gH+ complementing cell line. Samples of this virus stock are then used to prepare working stocks as before, and these working stocks are then used to infect laboratory animals as described below.

C. Studies on the Protective Effect of gH- HSV  
Compared to Heat Killed Virus

In order to assess the host immunological response to  
5 this virus, challenge experiments were conducted in  
mice according to the experimental plan described  
below.

The protective effect of a live gH<sup>-</sup> virus preparation  
10 was compared with an inactivated preparation of wild  
type (WT) virus (strain SC16) as follows.

Preparation of inactivated wild type virus for  
vaccination

15

HSV type 1 (strain SC16) was grown by low  
multiplicity infection (0.01 pfu/cell) of Vero  
cells. After three days, the virus was  
harvested, and cytoplasmic virus recovered by  
20 Dounce homogenisation. Nuclei were removed by  
centrifugation at 500xg for 15min, and the virus  
was recovered from the supernatant by  
centrifugation on to a 40% sucrose cushion at 12K  
for 60 min Beckman Sw27 rotor. The banded virus  
25 was diluted, pelleted and purified by sucrose  
gradient centrifugation (Killington and Powell,

1985, op. cit.). The virus band was harvested from the gradient, and the virus recovered by centrifugation. Virus was resuspended in phosphate-buffered saline (PBS), assayed for infectivity by plaque titration on baby hamster kidney (BHK) cells, and the particle count determined by electron microscopy. The particle:infectivity ratio of the preparation was 110 particles/pfu. The virus was diluted to  $2.5 \times 10^{10}$  pfu/ml in PBS, and inactivated by treatment with  $\beta$ -propiolactone for 60min at 20°C. Aliquots were then stored at -70°C.

#### Preparation of live gH<sup>-</sup> virus for vaccination.

This material was prepared as described for the wild type virus, except that the virus was grown in the gH<sup>+</sup> complementing cell line containing and expressing the HSV type 1 gH gene, and it was not inactivated by treatment with  $\beta$ -propiolactone. The particle: infectivity ratio of this preparation was 150:1. The concentration of this preparation was adjusted to  $2.5 \times 10^{10}$  pfu/ml, and aliquots were stored in PBS at -70°C.

25

Vaccination protocol

4 week-old female balb/C mice (purchased from  
Tucks U.K. Ltd) were vaccinated with various  
doses of inactivated WT virus or live gH- virus  
5 in 2 $\mu$ l volumes of phosphate-buffered saline by  
droplet application and needle scarification of  
the right ear as follows:

	Group A	Control - no virus
10	Group B	5 x 10 <sup>4</sup> pfu virus vaccine
	Group C	5 x 10 <sup>5</sup> pfu virus vaccine
	Group D	5 x 10 <sup>6</sup> pfu virus vaccine
	Group E	5 x 10 <sup>7</sup> pfu virus vaccine

15 After 14 days, all mice were challenged by similar  
inoculation of the left ear with 2 x 10<sup>6</sup> pfu HSV-1  
strain SC16 (wild type virus). Mice were killed after  
5 days and assayed for virus infectivity in the left  
ear and left cervical ganglia cII, cIII and cIV  
20 (combined). For latency studies, other vaccinated and  
challenged animals were killed after 1 month, and  
tested for latent infection by dissecting out the cII,  
cIII and cIV ganglia. These were incubated in medium  
for five days then homogenised and assayed for the  
25 presence of infectious virus by standard plaque assay.  
All the following results are expressed as pfu/organ.

Table 1- Titre of challenge virus present during the acute phase of infection after vaccination with live gH- virus

		Virus titre - log <sub>10</sub> pfu (WT SC16)			
	Mouse no.	Ears	mean	cervical ganglia*	mean
5					
10	group A	1	4.2	3.3	
		2	4.2	3.4	3.4
		3	4.6	3.4	
		4	4.3	3.4	
15	group B	1	3.4	1.5	
		2	none	2.4	1.8
		3	none	2.0	
		4	none	1.5	
20	group C	1	none	none	
		2	none	none	-
		3	none	none	
25		4	none	none	
30	group D	1	none	none	
		2	none	none	-
		3	none	none	
		4	none	none	
35	group E	1	none	none	
		2	none	none	-
		3	none	none	
		4	none	none	

40 \* Pooled cervical ganglia cII, cIII and cIV

Table 2- Titre of challenge virus present during the  
acute phase of infection after vaccination with  
 5 inactivated WT HSV-1

Virus titre - log <sub>10</sub> pfu (WT SC16)					
	Mouse no.	Ears	mean	cervical ganglia*	mean
10					
	group A	1	5.7	2.6	
		2	4.4	2.3	2.3
		3	5.7	2.1	
15					
	group B	1	4.2	1.9	
		2	3.6	3.1	1.2
		3	3.5	none	
		4	3.8	none	
20					
	group C	1	none	none	
		2	2.5	none	-
		3	2.9	none	
25		4	2.7	none	
	group D	1	3.9	none	
		2	2.0	none	-
30		3	2.0	none	
		4	2.3	none	
	group E	1	none	none	
35		2	none	none	-
		3	none	none	
		4	none	none	

40 \* Pooled cervical ganglia cII, cIII and cIV

Table 3- Titre of challenge virus present as latent virus  
in the cervical ganglia after vaccination with live gH-  
HSV-1

		mouse no.	Virus titre in- cervical ganglia* (log <sub>10</sub> pfu WT)	reactivation frequency
5				
10	group A	1	5.4	
		2	4.6	
		3	5.0	5/5
		4	4.8	
		5	5.3	
15				
	group B	1	none	
		2	1.5	3/4
		3	5.1	
20		4	5.3	
	group C	1	none	
		2	none	1/3
25		3	3.2	
	group D	1	none	
		2	none	0/4
30		3	none	
		4	none	
	group E	1	none	
		2	none	0/4
35		3	none	
		4	none	

40 \* Pooled cervical ganglia cII, cIII and cIV



Table 4 - Titre of latent challenge virus in the cervical ganglia after vaccination with inactivated WT HSV-1

5		mouse no.	Virus titre in-cervical ganglia* (log <sub>10</sub> pfu WT)	reactivation frequency
10	group A	1	none	
		2	5.0	
		3	5.0	3/4
		4	5.2	
15	group B	1	3.5	
		2	4.0	3/4
		3	5.5	
		4	none	
20	group C	1	3.6	
		2	5.1	2/4
		3	none	
		4	none	
25	group D	1	none	
		2	4.8	1/4
		3	none	
		4	none	
30	group E	1	none	
		2	none	0/4
		3	none	
35		4	none	

\* Pooled cervical ganglia cII, cIII and cIV

40 (p.f.u = plaque forming units; gH- is a virus with a defective gH gene).

These results show the titre of the challenge virus wt

SC16 present in the ears and cervical ganglia during the acute phase of infection. Thus, a low titre indicates good effectiveness of the vaccination regimen with gH- virus whereas a higher titre, 5 indicates poorer effectiveness. It is clear from the results that vaccination with live gH- HSV virus is very much more effective than an equivalent amount of inactivated WT virus. With the inactivated preparation, a dose of  $5 \times 10^7$  pfu was required to 10 prevent challenge virus replication in the ear, whereas with the live gH- virus, 100-1000 fold less virus was required. Live gH- virus vaccination with  $5 \times 10^5$  pfu and over, was also able to block replication of the challenge virus in the cervical ganglia during 15 the acute phase of infection, and furthermore showed a clear protective effect against the establishment of latent infection in the cervical ganglia.

HSV lacking the gH gene as a vector for immunisation  
20 against a foreign antigen: introduction of the gpl20  
gene of SIVmac strain 142 into the genome of gH- HSV  
virus

Viruses with deletions in essential genes may, as 25 described above, be used as safe vectors for the delivery of foreign antigens to the immune system, and

the gH- HSV virus described above provides a suitable example of a such a vector. This virus could be used to express any desired foreign antigen, but a particularly attractive possibility would be the major antigenic proteins of the AIDS virus human immunodeficiency virus (HIV). Thus these sequences would be inserted into the gH- HSV genome in a way that would ensure their expression during infection of normal cells (i.e. non-complementing cells) by the recombinant virus. Infection of an individual with such a virus could lead to a latent infection which, from time to time upon reactivation, would lead to a burst of production of the foreign antigen, resulting in stimulation of the immune response to that protein.

15

Since studies to test this approach directly in humans are not feasible at present, as an initial stage, the approach may be tested in monkeys using the Simian AIDS virus SIV<sub>mac</sub> (Simian immunodeficiency virus isolated from macaques). A suitable SIV gene for this purpose is that encoding the gp120 protein, one of the major antigenic targets for this virus. This gene is therefore introduced into the gH- HSV genome, and the efficacy of this virus as a vaccine to protect monkeys against challenge with SIV assessed.

25

The SIV gpl20 gene is first of all cloned next to the cytomegalovirus IE core promoter (Gompels and Minson, 1989, op. cit.), and subsequently a DNA cassette consisting of the gpl20 gene and the upstream CMV promoter is cloned into plasmid pGH2 (figure 2). The resulting plasmid is then co-transfected into the gH+ complementing cell line along with DNA purified from the gH- HSV, and recombinant virus which has acquired the gpl20 gene in place of the  $\beta$ -galactosidase gene present in the gH- HSV virus is isolated by screening for interruption of the  $\beta$ -galactosidase gene.

A. Construction of plasmid for recombination of the SIV gpl20 coding sequence into the HSV genome

The overall scheme for this procedure is shown in Figure 7. A SacI restriction enzyme fragment (corresponding to bases 5240-8721) is excised from a cloned DNA copy of the SIV genome (Chakrabarti et al., Nature 328, 543 (1987), and cloned into the SacI site of plasmid pUC118 (Viera and Messing, Methods in Enzymology, 153, 3, 1987) in order to generate plasmid pSIV1 which may be converted to single stranded DNA for manipulation by site directed mutagenesis. This DNA region, which includes the SIV env gene (lying between 6090-8298) is then altered by site directed

mutagenesis (Brierley et al., Cell 57, 537, 1989) to introduce a restriction enzyme site for the enzyme EcoRV at positions 6053-6058 using the synthetic oligonucleotide

5           5'GAAGAAGGCTATAGCTAATACAT.

A second EcoRV site is then introduced at position 7671-7676 within the SIV env gene corresponding to the cleavage site between the gp120 and gp40 domains of the env gene sequence, using the synthetic  
10   oligonucleotide

5'CAAGAAATAAACTATAGGTCTTTGTGC

to generate the plasmid pSIV2. A DNA fragment (1617 base pairs) corresponding to the gp120 portion of the SIV env gene is then prepared by digestion of SIV2  
15   with EcoRV.

The core region of the CMV immediate early gene promoter is obtained from the plasmid pUG-H1 (Gompels and Minson, 1989, op cit.) by the PCR technique using  
20   the following two synthetic oligonucleotides.

upstream primer

25           5' ATC GAATTC CTATAG CCTGGCATTATGCCCAGTACATG  
                  EcoRI       EcoRV

downstream primer

30           5'TCAAAGCTT CTATAG CCCGGGGGAGCTCTGATTATATAGACCTCCC  
                  HindIII   EcoRV           SmaI

The product of this reaction is then cleaved with the enzymes EcoRI and HindIII to generate a DNA fragment which is then cloned into EcoRI- and HindIII-digested plasmid pUC118 to generate the plasmid pCMVIE2 which  
5 has a unique SmaI site located just downstream of the CMV promoter sequence. The EcoRV fragment containing the SIV<sub>mac</sub> gp120 coding sequence prepared as described above, is then cloned into this SmaI site, and plasmid pSIV3, with the SIV coding region oriented correctly  
10 to allow expression of the coding sequence from the promoter, is then selected. This plasmid is then digested with EcoRV to yield a blunt-ended DNA fragment consisting of the SIV sequence together with the CMV promoter, which is then cloned into  
15 PvuII-digested pGH2 (Figure 2) to produce pGH-120.

B. Construction of the SIV gp120 carrying recombinant gH- HSV

20 DNA is purified from the gH- HSV virus constructed as detailed in the previous section, and co-transfected into gH+ complementing cells along with purified pGH-120 DNA. Progeny virus isolated from this transfection procedure is then plated on monolayers of  
25 the gH+ complementing cell line by standard plaque assay as before using an agar overlay in the presence

of X-gal. The parental gH- virus carries a functional  $\beta$ -galactosidase gene, located within the residual gH coding sequences, and in the presence of X-gal, will form blue plaques. Recombinant viruses however, which  
5 have acquired the SIV gp120 coding sequence in place of the  $\beta$ -galactosidase gene, will produce white plaques. Virus is recovered from these white plaques by picking plugs of agar, and virus stocks prepared through growth of the virus in the gH+ complementing  
10 cell line. Virus DNA is prepared from these stocks, and checked for the presence of the correct DNA structure around the gH gene by Southern Blotting using appropriate probes derived from the SIV coding sequence. Finally stocks of the virus are prepared as  
15 before for vaccination studies in animals.

A vaccine comprising the attenuated virus can be prepared and used according to standard techniques known in the art. For example, the vaccine may also  
20 comprise one or more excipients and/or adjuvants. The effective dose of the attenuated virus to be provided by the vaccine may be determined according to techniques well known in the art.

CLAIMS

1. A mutant virus whose genome is defective in respect of a gene essential for the production of infectious virus, and which is capable of protecting a susceptible species immunised therewith against infection by the corresponding wild-type virus.
2. A mutant virus according to claim 1 which is infectious for cells of a susceptible species immunised therewith.
3. A mutant virus whose genome is defective in respect of a gene essential for the production of infectious virus, and whose genome includes genetic material including an immunogenic protein from a pathogen exogenous to the virus.
4. A mutant virus according to claim 3 which is infectious for cells of a susceptible species, whereby the exogenous immunogenic protein is expressed in cells of said species infected with the mutant virus.
5. A mutant virus according to claim 3 or claim 4, wherein the immunogenic protein confers immunity against said pathogen in an infected species which is



normally susceptible to the pathogen.

6. A mutant virus according to claim 5 wherein the virus is capable in an infected species of establishing a latent infection with periodic reactivation.

7. A mutant virus according to any one of claims 3 to 6 wherein the exogenous protein is from an immunodeficiency virus.

8. A mutant virus according to claim 7 wherein the exogenous protein is an immunodeficiency virus glycoprotein.

9. A mutant virus according to any one of the preceding claims which is derived from herpes simplex virus (HSV).

10. A mutant virus according to claim 9 wherein the said defective gene is the HSV glycoprotein gH gene.

11. Use of a mutant virus according to any one of the preceding claims in the preparation of a vaccine.

12. A vaccine comprising a mutant virus according to

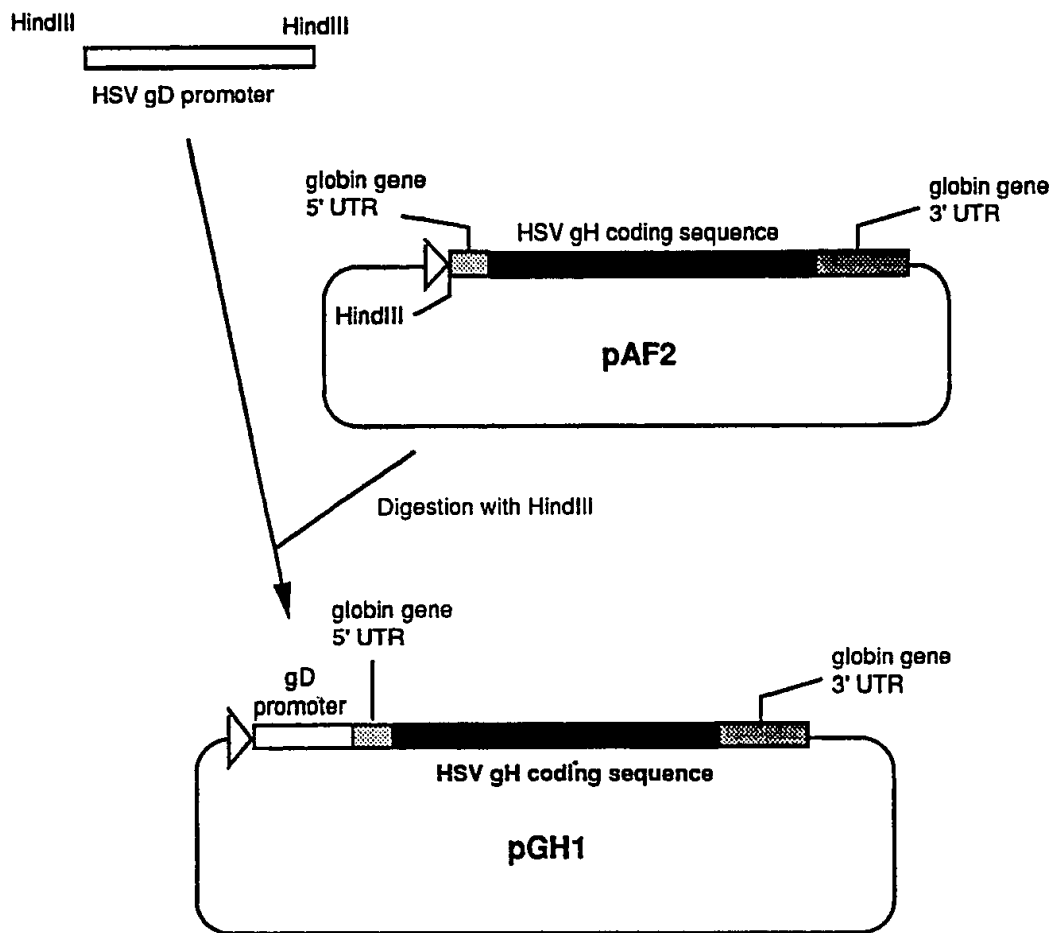
40

any one of claims 1 to 10.

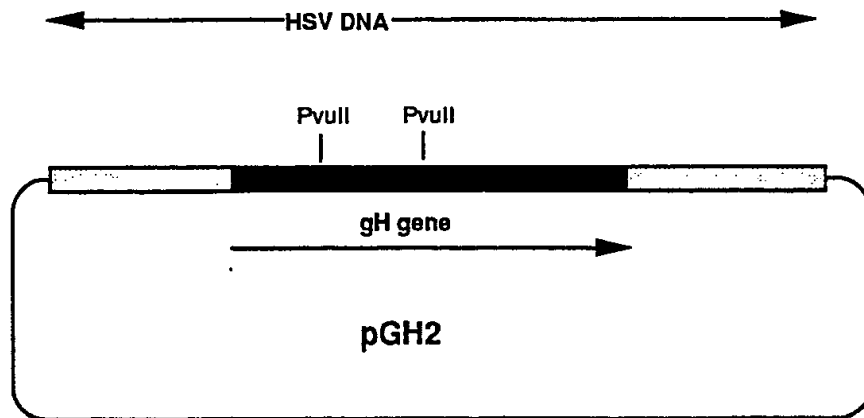
13. A method of making a mutant virus of any one of  
the claims 1 to 10, which comprises expressing in a  
5 recombinant host cell the genome of the virus which is  
defective in respect of said gene, the host cell  
expressing also a gene which complements the viral  
gene so as to allow the production of said mutant  
virus.

10

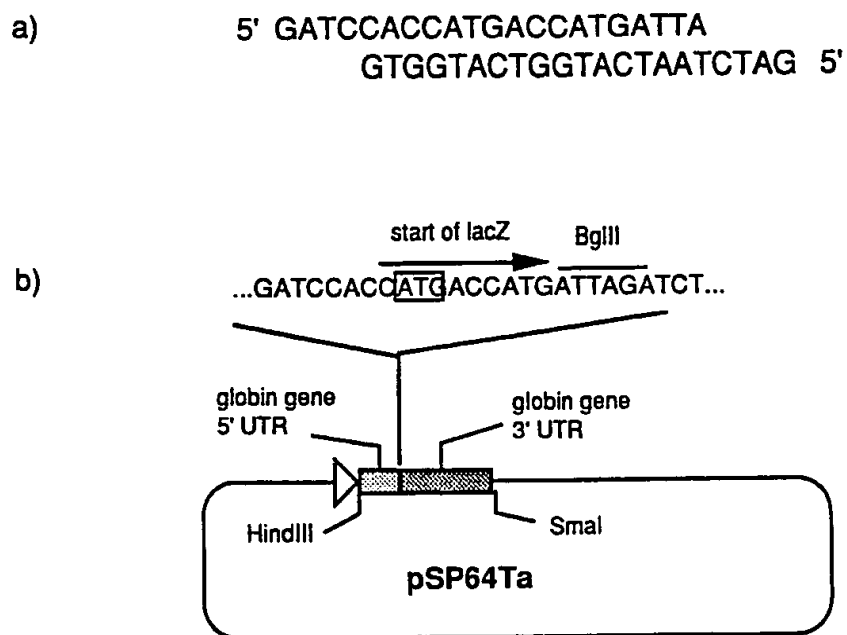
**Figure 1**



**Figure 2**



2/4

**Figure 3****Figure 4**

a) Upstream Primer

HindIII SmaI CMV sequence

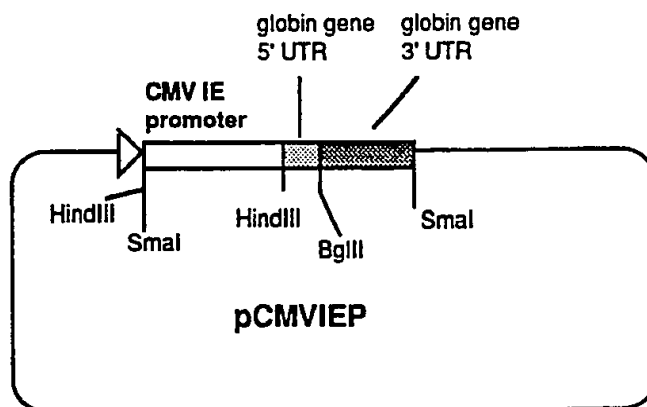
5' ATCAAGCTTCCCGGGCCTGGCATTATGCCAGTACATG

Downstream primer

HindIII CMV sequence

5' TCAAAGCTTGAGCTCTGATTATATAGACCTCCC

b)



3/4

Figure 5

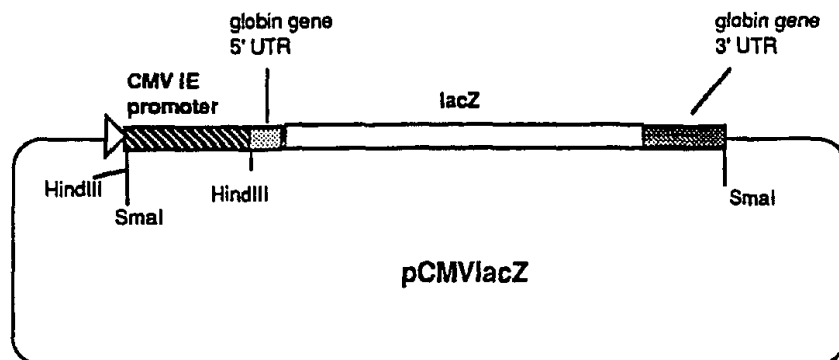
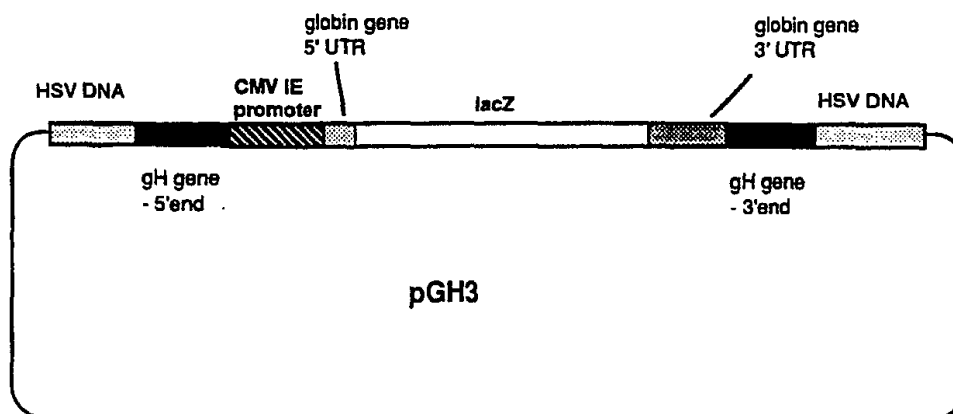
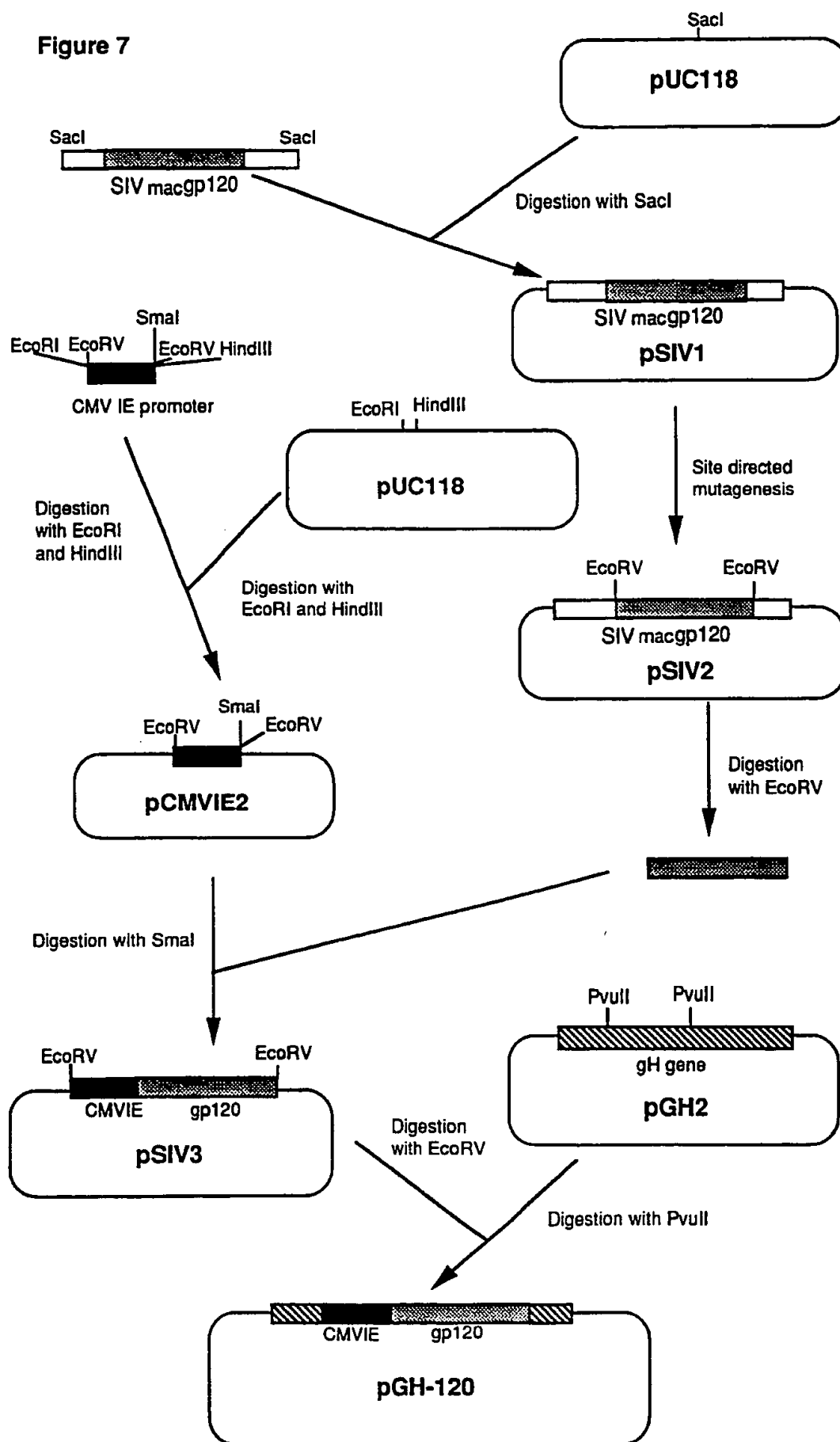


Figure 6



4/4

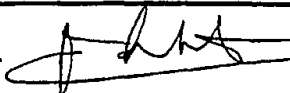
Figure 7



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 91/01632

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5	C12N15/86; C12N7/04	A61K39/235; A61K39/21; C12N7/01
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; C07K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	EP,A,0 213 894 (ADVANCED GENETICS RESEARCH INSTITUTE) 11 March 1987 see the whole document ---	1,2, 11-13
X	WO,A,9 005 538 (UNITED STATES OF AMERICA, THE SECRETARY, U.S. DEPARTMENT OF COMMERCE) 31 May 1990 see the whole document ---	1,2, 11-13
X	EP,A,0 386 882 (DANA FARBER CANCER INSTITUTE) 12 September 1990 see the whole document ---	1,2, 11-13
X	WO,A,8 909 271 (VIAGENE, INC.) 5 October 1989 see claims 1-7,29-34,41-43; figure 1 ---	1-8, 11-13
P,X	WO,A,9 105 055 (INRA) 18 April 1991 see claims 8-10,17-20 ---	1-2
-/--		
<sup>10</sup> Special categories of cited documents : <sup>10</sup> "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "A" document member of the same patent family		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
08 JANUARY 1992	16 JAN 1992	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	CHAMBONNET F.J. 	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claims No.
T	EP,A,0 453 242 (THE GENERAL HOSPITAL CORPORATION) ) 23 October 1991 see page 5, line 39 - line 47 ---	1
P,X	JOURNAL OF VIROLOGY. vol. 65, no. 3, March 1991, US pages 1202 - 1207; EMI, N. ET AL.: 'Pseudotype formation of Murine Leukemia Virus with the G protein of Vesicular Stomatitis Virus' see the whole document ---	1-5



**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. GB 9101632  
SA 51701**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 08/01/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0213894	11-03-87	AU-A- 6170486 JP-A- 62116523	26-02-87 28-05-87
WO-A-9005538	31-05-90	AU-A- 4661389 CA-A- 2002839 EP-A- 0441897	12-06-90 14-05-90 21-08-91
EP-A-0386882	12-09-90	CA-A- 2009403 JP-A- 3061492	06-08-90 18-03-91
WO-A-8909271	05-10-89	AU-A- 3364089 EP-A- 0334301 GB-A- 2236753 JP-T- 3504079	16-10-89 27-09-89 17-04-91 12-09-91
WO-A-9105055	18-04-91	FR-A- 2652504 AU-A- 6511890 EP-A- 0420759	05-04-91 28-04-91 03-04-91
EP-A-0453242	23-10-91	None	

